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Attorney's Docket No. **D0590/7003** (formerly B0192/7010)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Plaetinck et al.
Serial No	:	09/347,311
Filed	:	July 2, 1999
For	:	CHARACTERISATION OF GENE FUNCTION USING DOUBLE STRANDED RNA INHIBITION
Examiner	:	R. Shukla
Art Unit	:	1632

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to the Commissioner for Patents, Washington, D.C. 20231, on the 8th day of March, 2001.

Monica E. Zombori

Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith are the following documents:

[X] Letter to Commissioner
[X] Certified copy of priority document UK apl. no. 9814536.0, filed July 3, 1998
[X] Certified copy of priority document UK apl. no. 9827152.1, filed December 9, 1998
[X] Return Receipt Postcard

If the enclosed papers are considered incomplete, the Mail Room and/or the Application Branch is respectfully requested to contact the undersigned at (617) 720-3500, Boston, Massachusetts.

No check is enclosed. If a fee is determined to be required, the balance may be charged to the account of the undersigned, Deposit Account No. 23/2825. A duplicate of this sheet is enclosed.

Respectfully submitted,

By: John R. Van Amsterdam

John R. Van Amsterdam, Reg No. 40,212
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, MA 02210
Telephone (617) 720-3500

Docket No. D0590/7003
Dated: March 8, 2001
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Monica E. Zombori

Commissioner for Patents
Washington, D.C. 20231

LETTER TO COMMISSIONER

Sir:

Enclosed are certified copies of the two foreign priority documents listed below:

(1) UK apl. no. 9814536.0, filed July 3, 1998; and

(2) UK apl. no. 9827152.1, filed December 9, 1998

If any other information is needed, please contact the undersigned attorney by phone to expedite the further prosecution of this patent application.

Respectfully submitted,

By: John R. Van Amsterdam

John R. Van Amsterdam, Reg No. 40,212
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, MA 02210
Telephone (617) 720-3500

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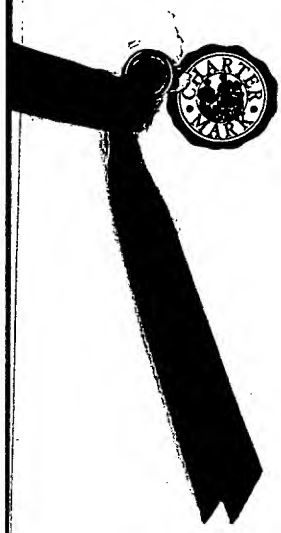

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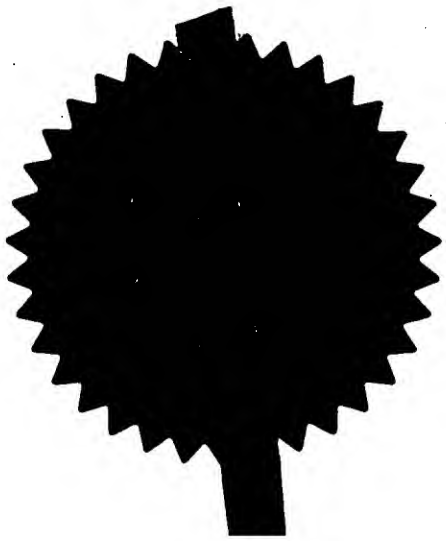

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1.	Your reference	SCB/50897/000
2.	Patent application number *(The Patent Off...*	9814536.0
		-3 JUL 1998
3.	Full ...me, address and postcode of the or of each applicant *(underline all surnames)*	**DEVGEN nv** **WOLVENDREEF 26g** **B 8500** **BELGIUM**
	Patents ADP number *(if you know it)*	7454911001
	If the applicant is a corporate body, give the country/state of its incorporation	**BELGIUM**
4.	Title of the invention	**CHARACTERISATION OF GENE FUNCTION USING DOUBLE STRANDED RNA INHIBITION**
5.	Name of your agent *(if you have one)*	**BOULT WADE TENNANT** **27 FURNIVAL STREET** **LONDON** **EC4A 1PQ**
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	
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CHARACTERISATION OF GENE FUNCTION USING DOUBLE STRANDED RNA INHIBITION

The present invention is concerned with characterization or identification of gene function using double stranded RNA inhibition (dsRNAi) and, in particular, with methods of identifying DNA responsible for inducing a specific phenotype in a cell and a method of assigning function to known gene sequences.

It has recently been described in Nature Vol 391, pp.806-811, February 98, that introducing double stranded RNA into a cell results in potent and specific interference with expression of endogenous genes in the cell and which interference is substantially more effective than providing either RNA strand individually as proposed in antisense technology. This specific reduction of the activity of the gene was also found to occur in the nematode worm Caenorhabditis elegans (*C. elegans*) when the RNA was introduced into the genome or body cavity of the worm either directly or by feeding the worm *E. coli* transformed with the or expressing the dsRNA corresponding to the gene of interest. In some cases, the reduction of activity lasted more than one generation.

The present inventors have utilized this technique and applied it further to devise novel and inventive methods of assigning functions to genes, which have been sequenced in various projects, such as, for example, the human genome project and which have yet to be accorded a particular function and for use in identifying DNA responsible for conferring a particular phenotype.

Therefore, according to a first aspect of the present invention there is provided a method of

identifying DNA responsible for conferring a desired phenotype in a cell which method comprises a) constructing a cDNA or genomic library of the DNA of said cell between two promoters capable of promoting transcription of said DNA to double stranded (ds) RNA upon binding of an appropriate transcription factor thereto, b)organising said library into a hierarchical pool , c) contacting a plurality of said cells adapted to express said transcription factor with said pool, and d) identifying and isolating a desired phenotype of said cell and identifying the DNA fragment from said pool responsible for said phenotype. Preferably said cell is derived from or contained in an organism.

The present invention also includes a method of validating yeast two hybrid vector experiments which experiments are well known to those skilled in the art and which experiments were first proposed by Chien *et al*. (1991) to detect protein - protein interactions. The method according to the invention comprises providing a construct including the DNA encoding a protein identified in a two hybrid vector experiment, which construct is such that said DNA is provided between two promoters capable of transcribing said DNA to double stranded RNA upon binding of an appropriate transcription factor thereto, transforming said construct into a bacterial or yeast cell expressing said transcription factor and identifying a phenotypic change in a model organism such as *C. elegans* or the like when the cell is introduced into said organism compared to wild type. Preferably, the construct is such that it may be used in the yeast two hybrid vector system experiment. When the construct is used in a yeast cell in accordance with the invention, the transcription factor may be inducible such that expression of the transcription factor capable of

inducing dsRNA repression during the yeast two hybrid experiment step does not occur. Thus, there is only a need to carry out one step to identify the protein of interest and to utilise the construct identified as having the necessary DNA to transform the subsequent bacterial or yeast cell. Also encompassed with the scope of the present invention are vectors using to transform said cells or organisms and the cells or organisms themselves.

According to a further aspect of the invention there is also provided A method of assigning function to a known gene sequence which method comprises a) identifying a homologue(s) of said gene in a model organism or cell, b) isolating the relevant homologue(s) DNA fragment from said cell or organism, c) cloning said fragment into an appropriate vector between two promoters capable of inducing transcription of dsRNA upon binding of an appropriate RNA polymerase thereto, d) introducing said vector into said cell or organism from step a) and which cell or organism is adapted to express said RNA polymerase, and e) identifying the phenotype of said cell or organism compared to wild type.

The present invention may be more clearly understood by the following examples which are purely exemplary with reference to the accompanying figures, wherein:

Figure 1 is a nucleotide sequence of plasmid PGN1 in accordance with the present invention.

Figure 2 is a nucleotide sequence of plasmid PGN100 in accordance with the present invention.

Figure 3 is a schematic representation of the vectors used and the transformation regime used in the methods according to the present invention.

Figure 4 is an illustration of the yeast two hybrid vector used in accordance with the invention.

Figure 5 is a schematic illustration of the T7 RNA polymerase expression vectors used for transforming *C. elegans*.

Figure 6 is a diagrammatic representation of plasmid PGN1.

Figure 7 is a diagrammatic representation of an enhanced vector for dsRNA inhibition encoding sup-35 dsRNA.

EXAMPLE A: Construction of an ordered and hierarchical pooled cDNA library and applications thereof.

<u>A random ordered and pooled library:</u>

The vector needs to have at least the following features: it is an *E. coli* vector harboring two T7 promoters, with a multiple cloning site (MCS) in between. The two promoters are orientated towards each other, and towards the MCS. In the presence of T7 RNA polymerase, expressed in *E. coli*, *C. elegans* or any other organism, RNA will be produced, starting from the two T7 promoters. As these are in opposite sense, both strands of RNA will be produced from the DNA inserted (cloned) into the MCS in between the two

promoters. The result of this expression of the two strands is the generation of double stranded RNA (dsRNA).

A *C. elegans* cDNA library is constructed in the MCS using standard molecular biological techniques. The library is transformed into *E. coli*, and the resulting *E. coli* are grown in culture and stored in 96 multi-well plates. At this stage, plasmid DNA can be isolated and stored in 96-multi-well plates corresponding to those of the *E. coli* colonies. Approximately 100,000 colonies are scored, in this way, the library will harbor approximately 5 times the total expressed cDNA variation of *C. elegans*, which gives the opportunity for low expressed sequences to be present in the library. This will result in approximately 1041 96-well plates. The plates are hierarchical pooled. Here several options are possible. This will be dependent on the reduction to practice. The present idea is that the pooling of the clones will happen in a range of 10 to 100. This may be dependent on the experiments. If the hierarchical pooling is per 8 or 12 (numbers are more convenient as 96-well plates have a 8 to 12 grind), this will result in approximately 87 multi-well plates and approximately 8352 wells. If hierarchical pooling is per 96 wells, which is a full plate, this results in approximately 11 plates and approximately 1041 wells. At any stage of the hierarchical pooling, plasmid DNA can be isolated, which would be less elaborate as less plates are used, but will result in a loss of complexity although this should not be the case in the pooling per 12. The pooling of the DNA can also be done with the original DNA.

The experiments described below, will help to decide how the hierarchical pooling should be

performed, both for the DNA as for the *E. coli* library.

An ordered library for RNAi technology, harboring every gene of the C. elegans genome, with applications thereof

We propose the construction of an ordered and hierarchical pooled library. As the genome-sequencing project is coming to an end, this information can be used in the T7 RNAi technology. Every gene of the *C. elegans* genome can be cloned using PCR technology. In preference, exons will be cloned with a minimal length of 500bp, if the exons are too small, smaller fragments will be isolated with PCR, or ever parts of interons and neighbor exons will be isolated with PCR technology. So that at least a sufficient part of the translated region of the gene is cloned. For this, at least 17000 PCR reactions need to be performed. This collection of PCR products will be cloned in a T7 vector as described (two T7 promoters oriented towards each other with a multiple cloning site in between). Every PCR product will be cloned independently, or can be used to generate a random library, analogous to the described cDNA library. If every PCR product is cloned individually, the resulting bacteria and plasmid DNA can be pooled in various ways. First of all this collection of individual cloned PCR products in the T7 RNAi vector can be pooled randomly, as described in the random library, but this pooling can also be done in a more rational way. For instance, the genes of the *C. elegans* genome can be analyzed using bioinformatic tools (in silico biology). Various genes of the genome will belong to a gene family, or will have homologues in the genome. These

members of the gene family will be pooled, or the members, being homologues will be pooled. In this way reducing the total number of about 17000 clones is reduced to a more useful quantity. This library can be used to screen for phenotypes in a RNAi experiment. The resulting phenotype gives a functional description to the gene or gene family or gene homologues of the *C. elegans* genome. As the library consists of a part of every gene in the genome, this method will enable us to describe the full genome in functional-phenotypic terms. For this the double stranded RNA (dsRNA) needs to be introduced in the worm. This introduction of clones alone, or pooled clones, being random pooling or rational pooling can be done on the several ways described and even on new ways to develop. We will use this T7 RNAi library for organized screening of phenotypes in the worm. Every gene can be analyzed in this way, every gene family can be analyzed in this way, and every homologue can be analyzed in this way. This project is in our view the best way to analyze in a rational way the full genome of the worm. Further combination can be made with mutant worms, and compounds.

Example of a vector for the expression of double stranded RNAi

Take any vector containing a T7 promoter, and a multiple cloning site (there are many commercially available). Design primers containing the T7 promoter and a primer with the reverse complementary strand, both with the appropriate ends. These primers can be hybridized, and if well designed, cloned in the vector of choice. The minimal sequence for a T7 promoter is TAATACGACTCACTATAGGGCGA. Although any vector can be

used for the construction of a T7 expression vector here is a detailed example of how to do this with pGEM-3Zf(-).

- Vector pGEM-3Zf(+) (PROMEGA) was digested with HindIII and SAlI
- Primers oGN1 and oGN2 were mixed together at a final concentration of 1 μg/30 μl boiled and cooled down slowly to room temperature.
- The primer was ligated into the vector using standard ligation procedures. The resulting vector is pGN1 (shown in Figure 1) and contains two T7 promoters oriented towards each other, and harbors a multiple cloning site in between.

Sequences of oGN1 and oGN2 are:

- oGN1: AGC TGT AAT ACG ACT CAC TAT AGG GCG AGA AGC TT
- oGN2: TCG AAA GCT TCT CGC CCT ATA GTG AGT CGT ATT AC

Example of the construction of a library

RNA may be isolated from every organism that is sensitive to RNAi. In general the isolated RNA is then copied into double stranded cDNA, and subsequently preparing the termini for vector cloning. Several procedures exist and molecular biology kits can be purchased from various firms including promega, clontech, boehringer Mannheim, BRL, etc.

- isolation of RNA,
- eventually polyA RNA can be isolated (several techniques and kits available)
- first strand synthesis with AMV reverse transcriptase, random hexameric primers and/or oligo(dT) primer
- second strand synthesis with Rnase H, DNA PolymeraseI,

- flush ends with T4 DNA Polymerase
- addition of an adaptor with T4 DNA ligase.
- eventually treatment with T4 polynucleotide Kinase
- cloning of the cDNA into the vector.

The resulting ligation mixture can be considered as the cDNA library. The ligation contains all cDNA of the procedure ligated into the vector of interest. To order the library, the ligation need to be transformed into E. coli strains.

Application of this library being *E. coli* or DNA

- T7 RNA producing strain:
- standard strain is BL21(DE3): F-ompT[lon]hsdS(r-m-; and *E. coli* B strain) λ (DE3). Eventually variants of BL21 (DE3) can be used, we are interested in BL21 (DE3)pLysS.
- any other *E. coli* strain which produces the T7 RNA polymerase, which may be available need to be constructed. This can be generated rather easily, using a phage, which is commercially available, in this case we will use the λCE6 (provided by Promega). Almost every *E. coli* strain can be transfected with this phage and will produce T7 RNA polymerase.
- a RNaseIII mutant *E. coli*:
- Various strains are in principle available, we chose in a first experiment to use strain AB301-105: rna-19, suc-11, bio-3, gdhA2, his95, rnc-105, relA1, spoT1, metB1. (Kinder *et al.* 1973 Mol. Gen. Genet 126:53), but other strains may suit better. This strain will be infected with λCE6 and so a T7 producing variant will be constructed. In a further process we will look at the possibilities for other strains, if this seems necessary.

- Wild type *C. elegans* worms can be grown on the bacteria pools. The bacteria is expressing the T7 RNA polymerase. This results in large quantities of dsRNA in the gut of the *C. elegans*, which will diffuse in the organism and results in the inhibition of expression (as reported previously). This library can now be used for the screening of several phenotypes. This would be a much faster technique to detect relevant genes in certain pathways, than the known *C. elegans* technology. Moreover, if an interesting phenotype is found, the responsible gene can be cloned easily. Using the hierarchical pooling one can easily find in a second screen the relevant clone of the pool. The inserted DNA of this clone can be sequenced. This experiment results in genetic and biochemical DATA in one step.
- Wild type *C. elegans* strain can be combined with compounds, screening for phenotype, drug resistance and or drug sensibility,
- The *C. elegans* strain can be a mutant strain, screening for an enhanced phenotype, reduced phenotype, or a new phenotype.
- The *C. elegans* strain can be a mutant strain, and the library screen can be combined with compounds. So one can screen for drug resistance, drug sensibility, enhanced phenotype, reduced phenotype, or a new phenotype.
- The *E. coli* strain may be any T7 RNA polymerase expressing strain, like BL21 (DE3) but the formation of double strand RNA may be enhanced by using a special *E. coli* strain that is RNAseIII negative. RNAseIII recognizes specific loops in dsRNA. Eventually, an *E. coli* strain can be used that is deleted in other RNAses than RNAseIII or an *E. coli* can be used that is deleted in one or more RNAses.

- The expression of the T7 RNA polymerase in most known *E. coli* strains and constructs that are available to generate T7 RNA polymerase producing *E. coli* strains mostly consists of an inducible promoter. This way the production of the T7 RNA polymerase is regulated, and thus the production of the dsRNA. Advantageously, this feature can be used to "pulse" feed the *C. elegans* worms at specific stages of growth. The worms are grown on the non-induced *E. coli* strains. When the worm has reached the stage of interest, the T7 RNA production in the bacteria is induced. This allows the studying of the function of any gene at any point in the life cycle of the animal.

1) Screening the library for homologues of putative interesting human genes, and assign function to these genes.

Hundreds of genes have been isolated in various projects, being genomic projects, differential expressed arrays, hybridization studies, etc. The described cDNA library may provide a way to validate and or assign function to these genes in a fast and efficient manner. First of all the worm homologue or homologues or the genes need to be identified by bioinformatic tools (in silico biology). PCR primers are developed and the cDNA fragment is isolated using PCR technology. PCR can be performed on the hierarchical pools. The positive pool or individual wells harboring the bacteria that has the appropriate cDNA is fed to *C. elegans* and the phenotype is scored.

PCR can be performed on cDNA isolated from *C. elegans*. The resulting DNA can be cloned in the T7 vector and transformed in the dsRNA producing *E. coli*, on which the *C. elegans* worms are then fed. Depending on which way is faster and more reliable a choice needs to be made.

If the gene belongs to a gene family, the worm may need to be fed on a mixture of bacteria. Each of them harboring a part of the member of the gene family.

E. coli strains, growth conditions, combinations with compounds can be performed as described above.

If the library rational is used, in which all the genes of *C. elegans* are cloned in a organized and structured way, the *C. elegans* homologue and eventually the other homologues, orthologues, and members of the gene family can be traced back easily in the library using in silico biology. No PCR is involved in this step, and the bacteria and or DNA can be isolated on which the worm will be grown.

Examples of testing the various principles described

The idea of the series of experiments is to test both the RNAi vector, the various *E. coli* strains that have been constructed.

1) Construction of a test plasmid.

Any cDNA that gives a clear phenotype in the worm when knocked-out, or used in a RNAi experiment can be used. It is known that unc-22 is a good candidate, but a lot of other genes are possible. We chose for a sensitive system that can be used in a later stage. We tested the system with sup-35 in a pha-1 background. Exon 5 of the sup-35 was isolated by PCR and cloned in the T7 promoter vector pGN1. The resulting vector was designated pGN2. pha-1 (e2123) mutant worms cannot produce offspring at temperatures higher than 25°C. This is due to a developmental problem in embryogenesis. When sup-35 is knocked-out,

or inhibited in this strain, offspring may grow at this temperature. Combination of pha-1 mutant worms and sup-35 RNAi is a good system to validate the various options.

2) Testing the RNAi using an *E. coli* strain that produces dsRNA.

- pGN2 was introduced in *E. coli* strain BL21(DE3) and T7 RNA polymerase was induced with IPTG. *C. elegans* worms (pha-1 (e2123)) were inoculated on this bacteria, and grown at the restricted temperature of 25°C. As this mutant is an embryonic mutant at this temperature, no offspring will be observed. If the sup-35 gene is efficiently inhibited by the dsRNA present in the *E. coli*, offspring will be observed.
- pGN2 was introduced in *E. coli* strain AB301-105(DE3) and T7 RNA polymerase was induced with IPTG. *C. elegans* worms (pha-1 (e2123)) were inoculated on this bacteria, and grown at the restricted temperature of 25°C. As this mutant is embryonic mutant at this temperature, no offspring will be observed. If the sup-35 gene is efficiently inhibited by the dsRNA present in the *E. coli*, offspring will be observed.

3) Improving the worm strain for better uptake of dsRNA.

Before plating the pha-1 *C. elegans* on the *E. Coli* strain that produce the double stranded sup-35 RNA. The worm was mutagenised with EMS (Methane Sulfonic Acid Ethyl). The offspring of this mutagenised worm is than plated on the bacteria. The worm that feed on this bacteria that gives a larger offspring has a mutation that may be the improvement of dsRNA uptake, and can be used for further experiments.

EXAMPLE B: a Yeast two-hybrid-RNAi vector, with applications

A yeast two hybrid vector can be constructed harboring the two T7 promoters. The vectors can be designed to replicate both in yeast and in *E. coli*. In general cDNA libraries for Yeast two hybrid being are made in the Gal4 or LexA vectors. The library is constructed in vectors having the activation domain of one of these genes. A vector can easily be constructed that still can perform in the yeast two hybrid screen but also contains the two T7 promoters orientated to each other, with a cloning site in between. The structure of the plasmid will then be "backbone, (GAL4-T7), MCS, T7, backbone". A *C. elegans* cDNA library constructed in this vector can be used as a standard yeast two hybrid library in an experiment to isolate interacting proteins with a given protein. Once a clone is isolated, the plasmid can be introduced in an *E.coli* strain expressing the T7 RNA polymerase, and hence will produce dsRNA of the cloned fragment. The bacteria producing this dsRNA can be fed to the worm and phenotypes can be scored. As in Example A), this validation procedure for a newly isolated yeast two hybrid clone is remarkably shorter than the standard procedure, which requires PCR and or cloning steps, RNA experiments and or knock-out experiments. In most cases isolated clones are sequenced first, and on basis of the sequence, a decision is made to continue with further experiments. In the present invention every isolated clone can easily be introduced into the appropriate *E. coli* and fed to the worm. Validation is then done by phenotype analysis.

Examples of procedure

A yeast two hybrid or as performed using a known gene as bait and the newly constructed library as the target. Proteins coded by the clones in the target that interact with the bait protein, will result in positive yeast clones expressing the reporter molecule such as that can be observed by LacZ staining with X-gal. The plasmid coding for the target protein can be isolated directly from the yeast strain and introduced in *E. coli*. The *E. coli* may be a T7 RNA polymerase producing *E. coli*. In this case, double stranded RNA will be produced of the DNA cloned in the multiple cloning site of the vector. When this dsRNA is fed to the worm using the methods described previously, the gene will be inhibited in the worm, resulting in a particular phenotype.

- This yeast two hybrid vector can be used to construct an ordered and hierarchically pooled library as described in Example A).
- A yeast strain could be constructed that conditionally produces T7 RNA polymerase. After yeast two hybrid experiments, the expression of the T7 polymerase could be induced, resulting in the production of dsRNA in the yeast cell. Consequently the yeast could be fed to the worm. Some evidence is known that worm can feed on yeast, nothing is known about the stability of dsRNA in yeast.

Example C: Construction of a T7 RNA polymerase producing strain, with applications

1) General description of the method

A *C. elegans* strain can be constructed that expresses T7 RNA polymerase. The expression can be

general and constitutive, but could also be regulated under a tissue specific promoter, an inducible promoter, or a temporally promoter or a promoter that harbors one of these characteristics or combination of characteristics. DNA can be introduced in this *C. elegans* strain. This can be done either by injection, by shooting with particles, by electroporationor as aforementioned by feeding. If the DNA is a plasmid as described in Examples A) and B), that is a plasmid harboring a cloned cDNA fragment or a PCR fragment between two flanking T7 promoters, than dsRNA of this cDNA or PCR fragment is formed in the cell or whole organism resulting in down regulation of corresponding gene. The introduced DNA can have an efficient transient down regulation. The introduced DNA can form an extrachromosomal array, the array might result in a more catalytic knock-out or reduction of function phenotype. The plasmid might also integrate into the genome of the organism, resulting in the same catalytic knock-out or reduction of function phenotype, but stably transmittable.

- Plasmid DNA harboring a cDNA or a part of a cDNA or an EST or an PCR fragment of *C.elegans* cloned between two T7 promoters as described in Examples A) and B) can be introduced in the T7 RNA polymerase worm, by standard techniques. Phenotypes can be analysed -DNA from an ordered and pooled library as in Example A) can be introduced in the T7 RNA polymerase worm, by standard techniques (injection, shooting). Phenotypes can be analysed. With the hierarchical pool, the original clone can be found easily.
- The same procedure can be done with a mutant worm expressing the T7 RNA polymerase. Screening for enhanced, reduced or new phenotypes.
- The procedure can be combined with compounds.

Screening with either a wild-type strain or a mutant strain for enhanced or new phenotypes.
- The DNA could be introduced in the worm by new methods. One of which is the delivery of DNA by *E.coli*. In this case the hierarchical pooled library is fed to the animal. To prevent digestion of the *E, coli* DNA in the gut of the nematode, preferentially a DNAse deficient *C.elegans* will be used, such as nuc-1 (e1392). This procedure would be one of the most interesting. As it would be independent of transformation efficiencies of other techniques, and at least faster and less labourious.

2) Putative enhancements of the method. (eventually also applicable to Examples A) and B).
- A vector is designed, so that it harbors the sup-35 cDNA or a part of this cDNA, cloned in between two T7 promoters. The rest of the vector is as described in Examples A) and B). This vector can be introduced into a pha-1ts mutant *C.elegans*. A temperature selection system exists in this case and only those worms which have taken up the DNA and express the double stranded sup-35 RNA will survive at restricted temperatures. The hierarchical pooled library can be delivered by any method described above.
- The vector can be used to construct a library that is introduced in a T7 RNA polymerase expressing *E. coli*. In this case we have an analogous screening as in part A)with an additional screening for worms where the dsRNA of sup-35 is active.
- The DNA and or dsRNA of sup-35 could be delivered on a different plasmid. For the feeding, both DNA feeding (Example C) or dsRNA feeding Example A) and B), this means that the two plasmids could be present in one bacterium, or that the worm is fed on a mixture of

bacteria, one of which harbors the sup-35 construct.

Example of the construction of a T7 RNA producing C. elegans

To produce T7 RNA polymerase in the worm, several possibilities are possible. The T7 polymerase can be expressed under various promoters, being inducible promoters, constitutive promoters, general promoters and tissue (cell) specific promoters, or combinations of those. Examples of these promotes are the heatshock promoter hsp-16, the gut promoter ges 1, but also the promoter of dpy7 and the promoter element GATA1. In this example the T7 RNA polymerase is expressed under the control of the hsp-16 promoter that is available in the pPD49.78 vector. The T7 RNA polymerase is isolated as a PCR product using the primers of oGN3 an oGN4. Procedures as in standard PCR technology.

The resulting PCR product is digested with NheI and NcoI, as is the vector in which we want to clone, being the Fire vector pPD49.78. The resulting vector is pGN100 illustrated in Figure 2 oGN3: CAT GGC AGG ATG AAC ACG ATT AAC ATC GC oGN4: ATG GCC CCA TGG TTA CGG GAA CGC GAA GTC CG pGN100 is included.

The vector is introduced into the worm using standard techniques, like micro injection .

The following strains were then constructed:

-Wild-type (pGN100)
-nuc-1 (e1392)(pGN100)
-pha-1 (e2123)(pGN100)
-pha-1; nuc-1 (pGN100)

All these strains are able to produce T7 RNA

polymerase when temperature induced. The procedure for induction is to shift the animal to 30-33°C for at least one hour, then the animal can be shifted back to standard temperatures (15-25°C).

The wild type strain producing T7 RNA polymerase can be used for the production of any RNA in the worm. More specifically, the plasmids from the described libraries can be introduced in these worms, and phenotypes can be scored.

The nuc-1 mutant worm will be used to introduce DNA via bacteria on which the worm feed. As the nuc-1 worm does not digest the DNA, the plasmid DNA can cross the gut wall. If taken up by the cells that produce the T7 RNA polymerase, dsRNA will be produced and inhibiting the gene for which the RNA transcribed.

The pha-1 mutant strain that produced T7 RNA polymerase can be used to enhance the procedures as described above, DNA can be introduced by shooting, micro injection or feeding. More specifically this strain can be used for the vectors that produce dsRNA from sup-35 and from the gene of interest, the later can be a PCR product, a cDNA, or a library as described.

The pha-1; nuc-1 mutant producing T7 RNA polymerase can be used for the bacterial delivery of the DNA. DNA will be preferentially the plasmid that produce dsRNA from both sup-35 and the gene of interest. The worm strain will preferentially produce the T7 RNA polymerase in the gut. Delivery will preferentially happen by feeding the worm on bacteria harboring the plasmid.

Application of the RNAi technology in plants

Nematodes consist of a large part of the damage

done towards plants and more particularly to plants used in the agricultural industry. We propose the use of RNAi to prevent these parasitic nematodes from feeding longer on these plants. In a first step, a DNA fragment need to be isolated from the parasitic plant nematode that the animal needs survive or to grow, or to feed or to proliferate. Any gene from which the expression is essential can be suitable for this purpose.

A part of this gene, an exon or cDNA is cloned. This DNA fragment can be cloned under the influence of a root specific promoter, or between two root specific promoters. The DNA of the cloned gene under the control of the root specific promoter can be introduced in the plant of interest, using the standard technology of making plant transgenics. For every parasitic nematode, a different piece of DNA can be needed, for every plant race, a different promoter can be needed.

The root will produce RNA or dsRNA from the introduced piece of DNA. As the nematode feeds on the plant, also the RNA and/or dsRNA will be eaten. The RNA and/or dsRNA can enter the cells of the nematode and perform its action of inhibition. Depending on the nature of the cloned DNA piece of worm, the nematode will not be able to survive, to eat, proliferate, etc in any case preventing the animal of feeding longer on the plant, and thus protecting the plant.

Claims

1. A method of identifing DNA responsible for conferring a particular phenotype in a cell which method comprises

a) constructing a cDNA or genomic library of the DNA of said cell between two promoters capable of promoting transcription of said DNA to double stranded (ds) RNA upon binding of an appropriate transcription factor thereto,

b) organising said library into a hierarchical pool ,

c) contacting a plurality of said cells adapted to express said transcription factor with said pool, and

d) identifying and isolating a desired phenotype of said cell and identifying the DNA fragment from said pool responsible for said phenotype.

2. A method according to claim 1 wherein said cell is derived from or contained in an organism.

3. A method according to claim 2 wherein said organism is adapted to express said transcription factor.

4. A method according to claim 2 or 3 wherein said organism comprises a plant, animal, fungus or yeast and preferably the nematode worm *C. elegans*.

5. A method according to claim 4 wherein said DNA library is transformed in a bacterial or yeast cell for introduction into said organism.

6. A method according to claim 5 wherein said yeast or bacterial cell is adapted to express said transcription factor.

7. A method according to claim 4 wherein said bacterial cell is *E. coli*.

8. A method according to any preceding claim wherein said promoter is an inducible promoter.

9. A method according to any of claims 1 to 8 wherein said cell or organism is contacted with a specified compound for screening of a desired phenotype such as resistance or sensibility to said compound.

10. A method according to any of claims 1 to 9 wherein said transcription factor is a phage derived RNA polymerase such as T7 polymerase.

11. A method according to any of claims 7 to 10 wherein said *E. coli* strain is an RNAase and preferably an RNAase III negative strain.

12. A method of assigning function to a known gene sequence which method comprises

a) identifying a homologue(s) of said gene in a model organism or cell,

b) isolating the relevant homologue(s) DNA fragment from said cell or organism,

c) cloning said fragment into an appropriate vector between two promoters capable of promoting transcription of dsRNA upon binding of an appropriate transcription factor thereto,

d) introducing said vector into said cell or

organism from step a) and which cell or organism is adapted to express said RNA polymerase, and

e) identifying the phenotype of said cell or organism compared to wild type.

13. A method according to claim 12 wherein said model organism comprises *C. elegans*.

14. A method according to claim 12 or 13 wherein said vector is transformed, transfected or infected into a yeast or bacterial cell adapted to express said polymerase and which bacterial or yeast cell is fed to said model organism.

15. A method according to any of claims 12 to 14 wherein said organism is *C. elegans*.

16. A method of validating clones identified in yeast two hybrid vector experiments which method comprises

a) providing a construct including the DNA encoding the protein identified in the two hybrid experiment, which construct is such that said DNA is provided between two promoters capable of transcribing double stranded RNA upon binding of an appropriate transcription factor thereto,

b) transforming said construct into a cell, such as a bacterial or yeast cell, expressing said transcription factor, and

c) identifying a phenotypic change in said cell as compered to a wild type.

17. A method according to claim 16 wherein said construct is such that it may be used in the yeast two hybrid experiment.

18. A method according to claim 17 wherein said transcription factor is inducible in said yeast cell subsequent to said two hybrid vector step.

19 A method according to any of claims 16 to 18 wherein said promoter is a phage polymerase promoter and said transcription factor is a RNA polymerase, and preferably T7 polymerase.

20. Plasmid pGN1 as illustrated in Figure 1.

21. Plasmid pGN100 as illustrated in Figure 2.

22.The yeast two hybrid vector plasmid illustrated in Figure 4.

23. A Plasmid as illustrated in Figure 7.

24. An organism or cell transformed or transfected according to any of the methods of claims 1 to 19.

25. A DNA or RNA molecule identified or produced according to any of the methods of claims 1 to 19.

26. A DNA or RNA molecule according to claim 25 for use as a medicament.

pGN1

gagtgcaccatatgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgcatcaggcgaaattgtaaacgttaatattt
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Figure 1

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Figure 1

PGN100
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Figure 2

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Figure 2

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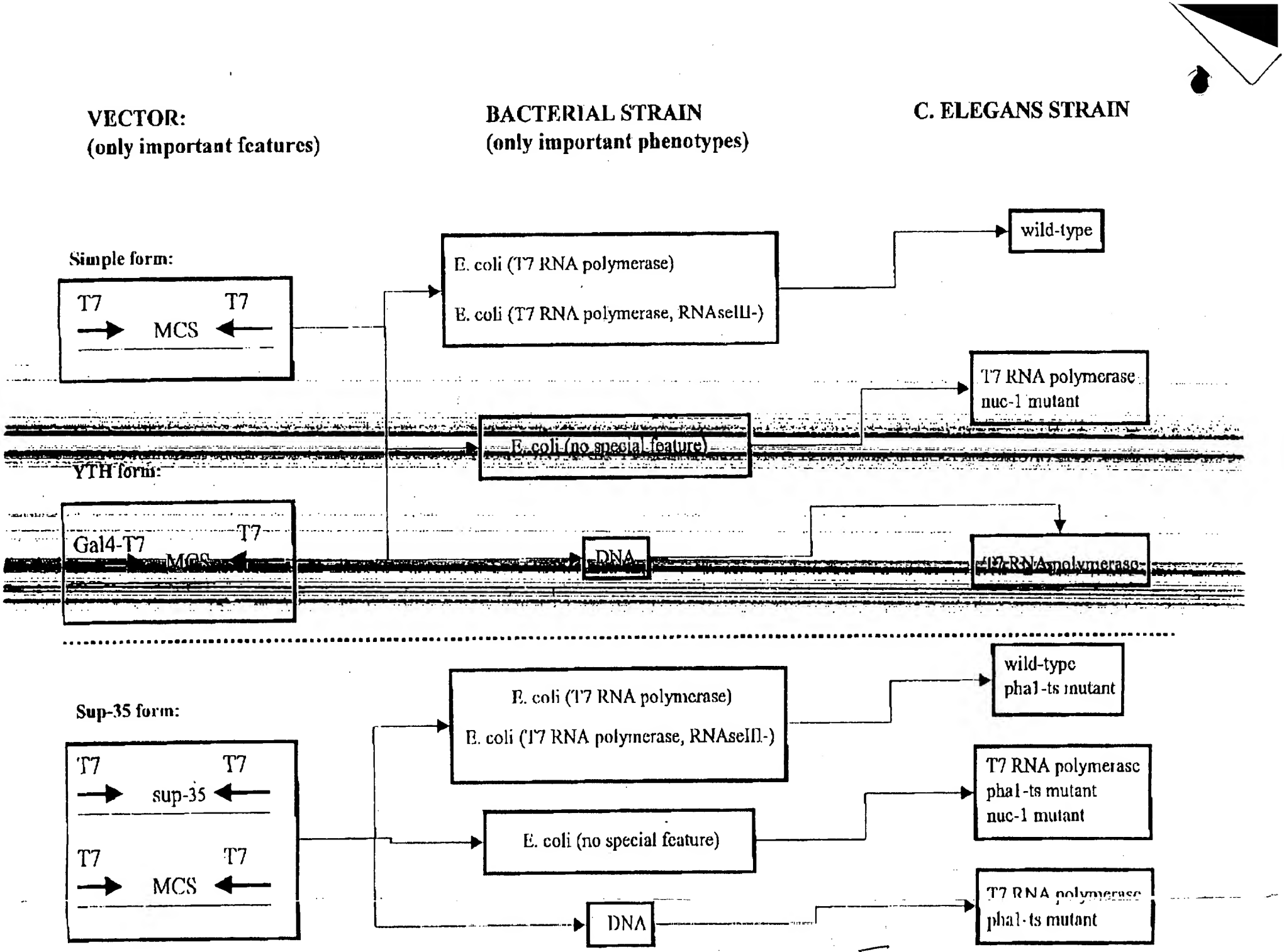
Figure 2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(only important features)
BACTERIAL STRAIN
(only important phenotypes)
C. ELEGANS STRAIN
Simple form:
T7
T7
MCS
E. coli (T7 RNA polymerase)
E. coli (T7 RNA polymerase, RNAseIII-)
wild-type
T7 RNA polymerase
nuc-1 mutant
E. coli (no special feature)
YTH form:
Gal4-T7
T7
MCS
DNA
T7 RNA polymerase
Sup-35 form:
T7
T7
sup-35
T7
T7
MCS
E. coli (T7 RNA polymerase)
E. coli (T7 RNA polymerase, RNAseIII-)
wild-type
phal-ts mutant
E. coli (no special feature)
T7 RNA polymerase
phal-ts mutant
nuc-1 mutant
DNA
T7 RNA polymerase
phal-ts mutant

Figure 3

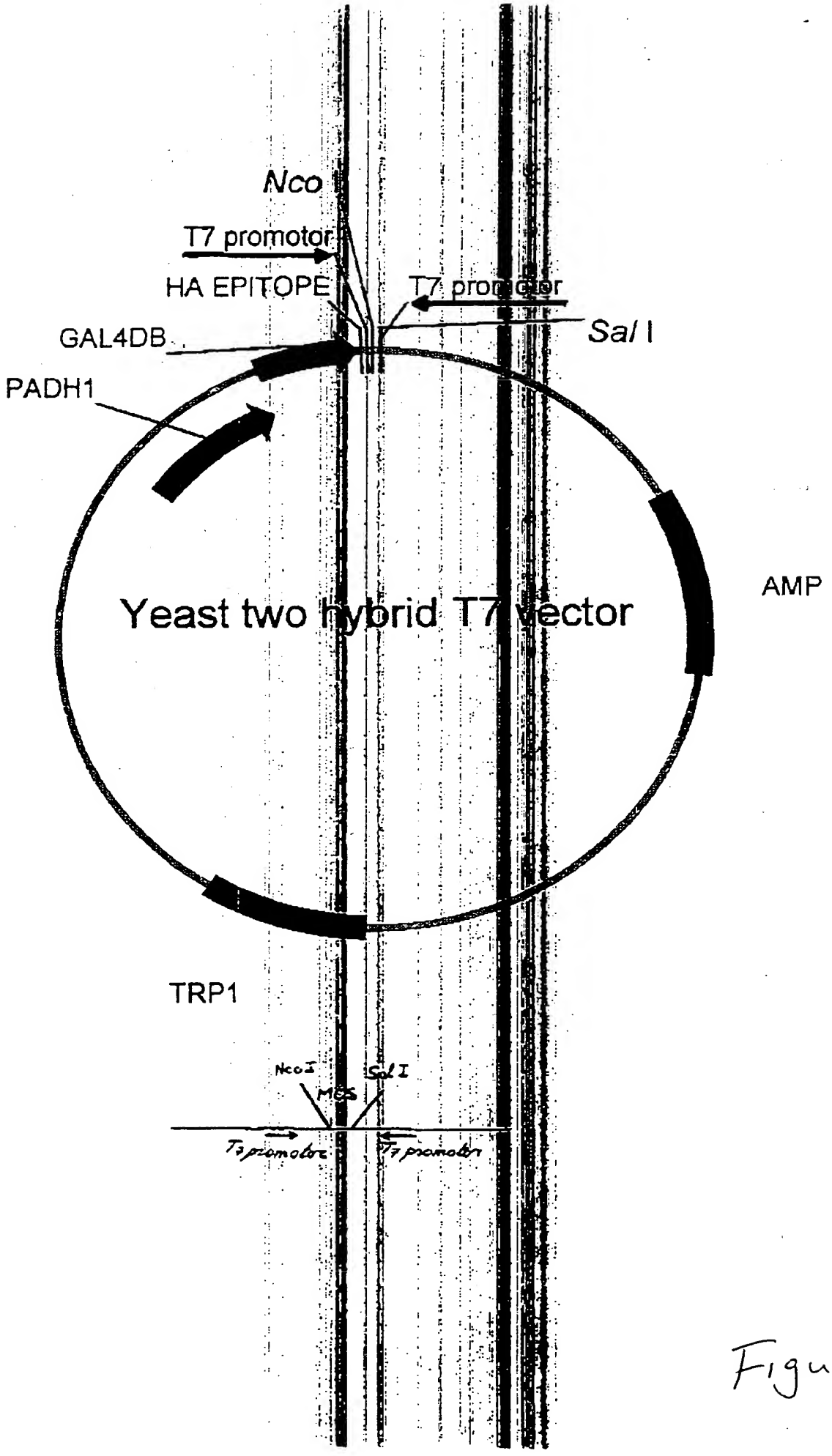

Nco
T7 promotor
HA EPITOPE
T7 promotor
Sal I
GAL4DB
PADH1
Yeast two hybrid T7 vector
AMP
TRP1
NcoI
MCS
SalI
T3 promotor
T7 promotor

Figure 4

General description of the C.elegans T7 RNA polymerase expression vector with 4 examples

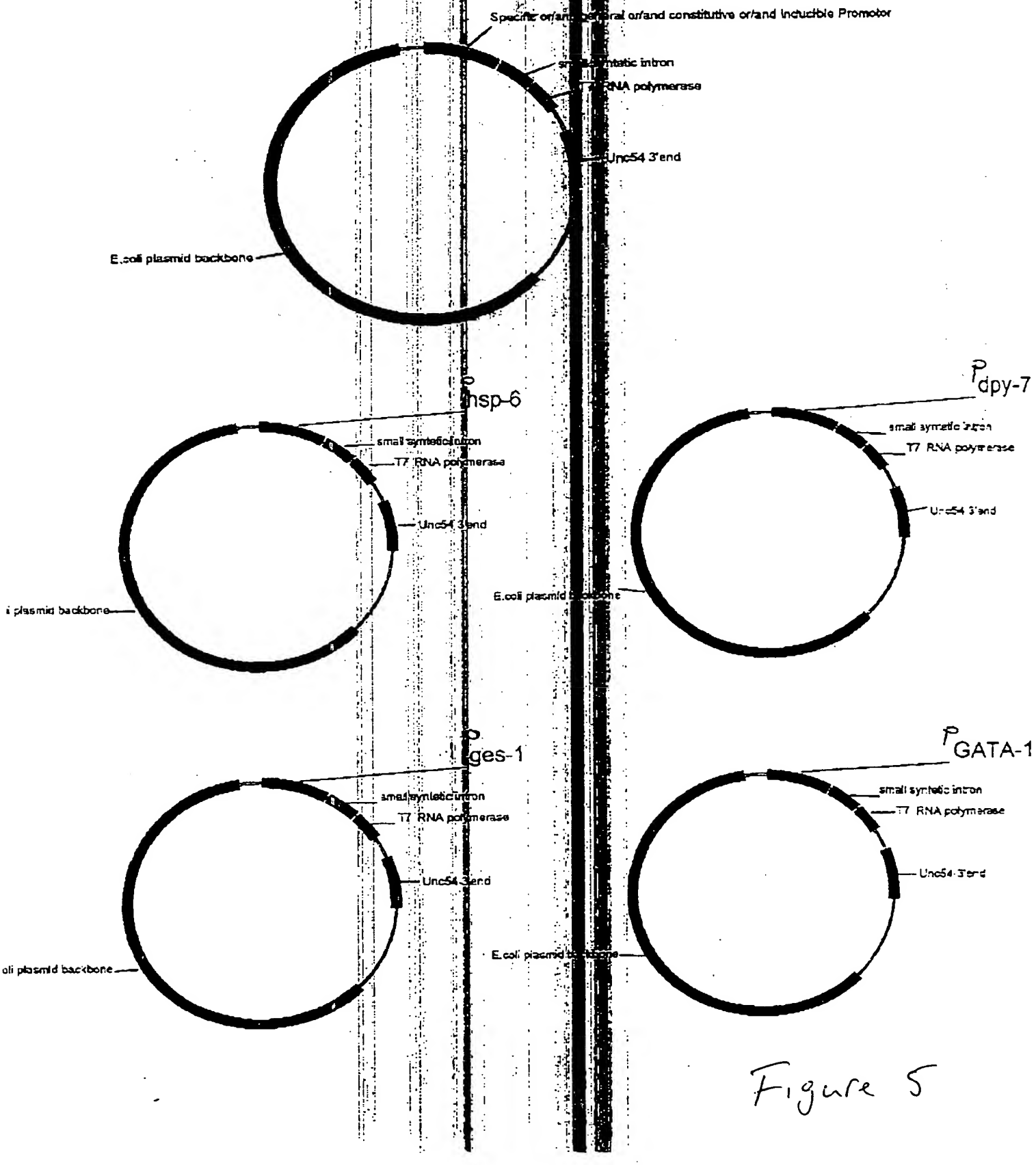


Figure 5

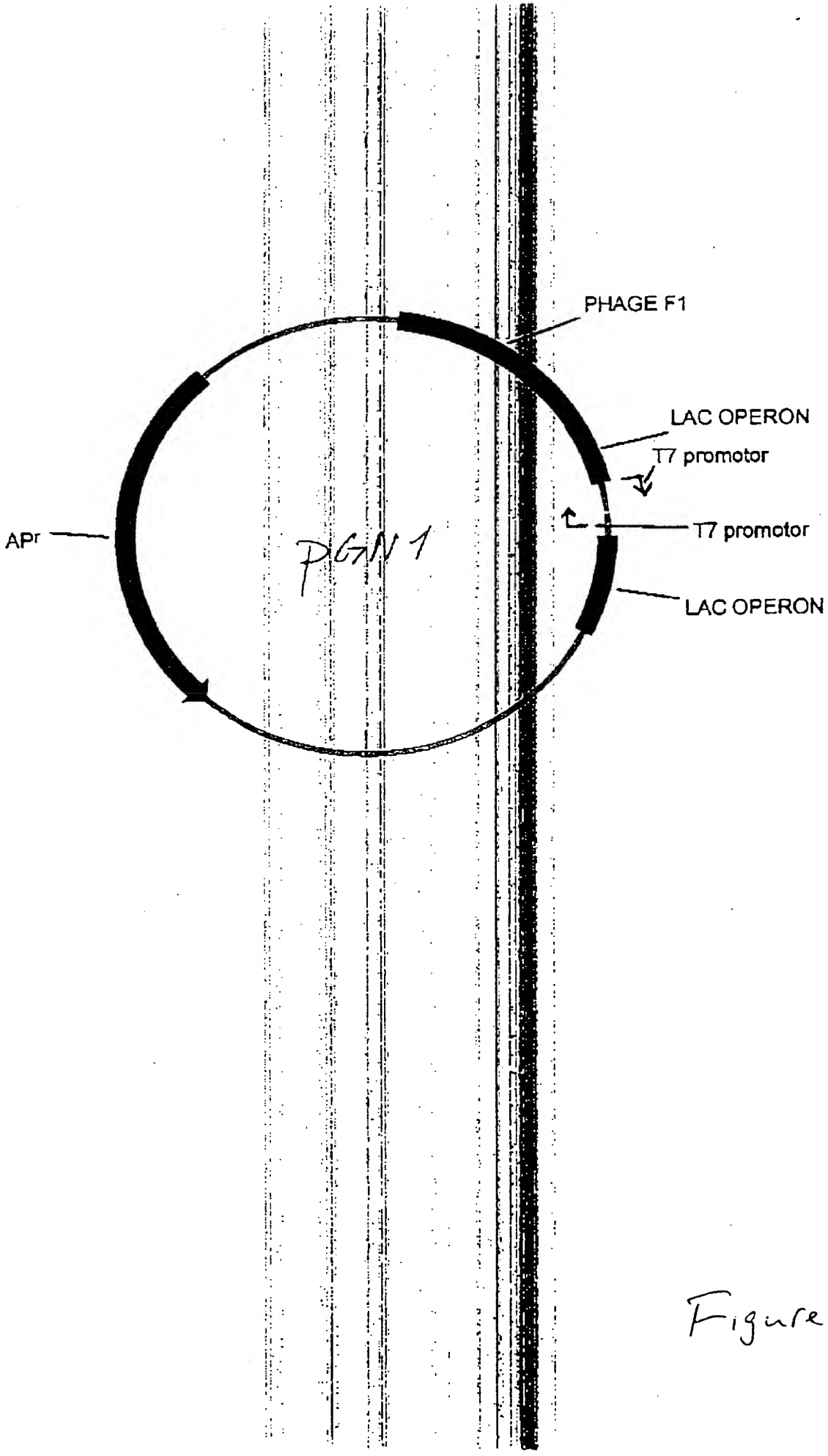
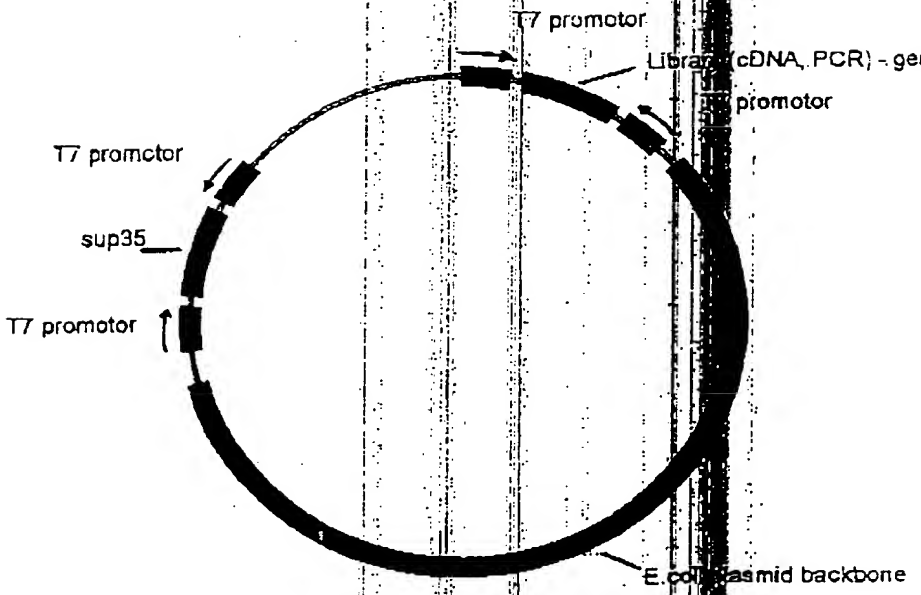

PHAGE F1
LAC OPERON
T7 promotor
T7 promotor
APr
PGN 1
LAC OPERON

Figure 6



enhanced vector for RNAi, producing sup35 ds RNA and ds RNA of the library, gene of interest or PCR product

Figure 7